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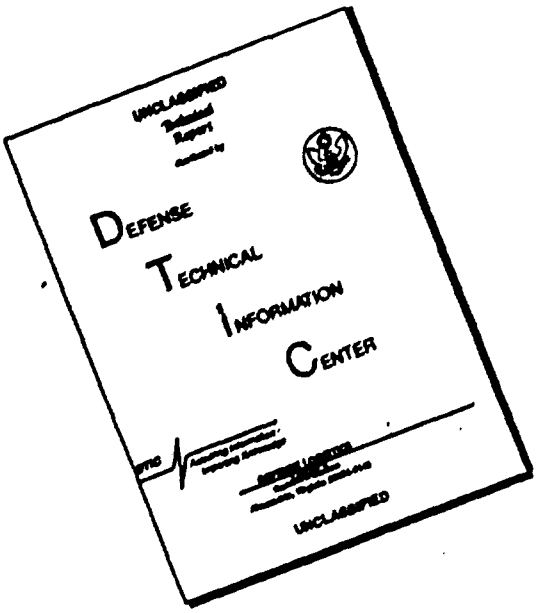

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Ethane Production in Copper-deficient Rats (43114)

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Abstract. **Evidence is accumulating which indicates that copper-deficient animals are prone to oxidative damage. To investigate this possibility further, we measured the production of breath ethane, a hydrocarbon by-product of lipid peroxidation, in copper-deficient rats. Male, weanling Sprague-Dawley rats were fed either a purified diet which was deficient in copper (CuD) or the same diet made sufficient with 5 ppm of copper (CuS). After 33 to 34 days the rats were placed individually in gastight metabolic cages through which ethane-free air or 100% O_2 was passed. Expired ethane was absorbed onto cold, activated charcoal, liberated by heating, and measured by gas chromatography. Ethane production rates (pmoles/min/100 g ± SD) were 3.3 ± 0.8 (CuS-air), 4.3 ± 1.4 (CuD-air), 8.3 ± 2.5 (CuS-O_2), and 12.2 ± 4.3 (CuD-O_2). Repeated measures analysis of variance indicated that both copper deficiency ($P < 0.01$) and breathing 100% O_2 ($P < 0.0001$) enhanced ethane production, with no interaction between treatments. This finding complements previous evidence that increased lipid peroxidation occurs in copper-deficient rats.** [P.S.E.B.M. 1990, Vol 195]

Because enzymes which may be regarded as having antioxidant function (e.g., superoxide dismutase, ceruloplasmin, catalase, cytochrome oxidase) are reduced in copper deficiency (1–3), it is reasonable to suggest that copper-deficient animals may be more susceptible to damage by the free radicals produced by metabolism. Evidence is accumulating to support this suggestion. Paynter (4) and Fields *et al.* (5), using free radical-generating systems, showed that tissues of copper-deficient rats are more prone to oxidative damage than those of copper-sufficient animals. Balevska *et al.* (6) measured higher lipid hydroperoxide levels in liver fractions of copper-deficient rats than in those of copper-sufficient rats. Saari and Johnson (7, 8) have shown that the cardiovascular effects of copper deficiency can be prevented partially by the feeding of exogenous antioxidants.

The above studies, except for that of Balevska *et al.* (6), have provided only indirect evidence that increased peroxidation actually occurs in copper deficiency. Ethane is a volatile by-product of the oxidation of n-3 fatty acids (9), is regarded as a reliable index of the degree of ongoing lipid peroxidation (10, 11), and is readily measured in the exhaled breath of rodents (12–16). The object of the present study was to utilize breath ethane measurements to determine in a relatively direct fashion whether lipid peroxidation was increased by copper deficiency in living animals.

Materials and Methods

Animals and Diets. Twenty male, weanling Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI)[1] were fed *ad libitum* a purified diet (Table I) which was either deficient in copper (CuD, $n = 10$) or was made sufficient with 5 μg of copper/g of diet (CuS, $n = 10$). Both groups received deionized water *ad libitum*. All animals were housed in quarters maintained at 22–24°C with a 12-hr light-dark cycle. After 32 days on their respective diets, the two groups of animals were shipped overnight in separate containers from Grand Forks to Tucson. One copper-deficient animal died en

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route. After a 1-day stabilization period, the breath ethane measurements were begun.

Measurement of Breath Ethane. Ethane production rates were determined as described previously (14–16). Each rat was housed in a metabolic chamber through which a unidirectional flow of either ethane-free air (Liquid Air, Tucson, AZ) or 100% oxygen (Liquid Air) was directed at 200 ml/min. The inflowing gas was directed through a cold trap of freshly prepared (vacuum-heated to 240°C for 1 hr) activated charcoal (Alltech) to absorb hydrocarbon in the incoming gas. Effluent from the chamber was directed via Teflon tubing to a small glass cassette of activated charcoal placed in a second cold trap. This cassette was replaced at 30-min intervals, and the charcoal contents of each cassette were poured into a glass test tube of known volume and sealed with an open screw top containing a Teflon septum. This tube was heated at 240°C for 5 min to liberate the ethane. Five milliliters of headspace gas was then removed from this test tube using an air-tight gas sampling syringe and injected onto the column of a gas chromatograph (model 5890; Hewlett-Packard, Palo Alto, CA) maintained isothermic at 220°C.

The chromatograph column was a 2-m long, 0.25-inch diameter glass column packed with Carbosphere 60/80 (Alltech). Output was recorded on an integrator (Hewlett-Packard model 3393A) connected in line with the chromatograph. Calibration of the chromatograph was performed the morning before each study using known quantities of pure ethane gas standard. The resulting calibration curves were linear with regression coefficients not less than 0.995. Retention time for ethane was 4.74 min under the conditions used.

Rats were allowed deionized water only for 8 hr before the study. The animals were exposed to ethane-free air in the chambers for 2 hr (four samples taken at 30-min intervals), then the inspirate was changed to 100% oxygen and, after a 15-min washout period, ethane collections on oxygen were performed, again over a 2-hr period.

The ethane from the 5-ml headspace determination was used to determine the total headspace ethane content. The total ethane content so determined from each 30-min collection period was cumulated for 2-hr for each animal and expressed per 100 g of body wt. The least squares linear regression line was calculated for a plot of cumulated ethane (expressed in nanomoles/100 g vs time in minutes). The slope of the regression line represents the ethane production rate for that animal under the given test conditions, those conditions being breathing either ethane-free air or oxygen.

Blood, Organ, and Diet Analysis. Following breath ethane measurements, each rat was anesthetized, blood was withdrawn and centrifuged for serum, and the liver, lung, heart, and one kidney were removed.

Table I. Diet Composition

Ingredient	Amount (g/kg)	
Basal diet[a]	940.0	
Safflower oil[b]	50.0	
Ferric citrate·*n*-hydrate (16% iron)[c]	0.22	
	CuS (g/kg)	CuD (g/kg)
Cornstarch[d]	9.76	9.78
Cupric sulfate pentahydrate[c]	0.02	0

[a] A casein (20%), sucrose (39%), cornstarch (29%)-based diet containing all known essential minerals and vitamins except iron and copper (Teklad Test Diets, Madison, WI; catalog no. TD 84469). See Ref. 17 for exact composition.
[b] Hollywood Foods, Los Angeles, CA.
[c] J. T. Baker Chemical Co., Phillipsburg, NJ.
[d] Best Foods, Englewood Cliffs, NJ.

Serum and organs were frozen and stored until shipped on dry ice from Tucson to Grand Forks.

Serum was analyzed using a Cobas fara automated analyzer (Roche Diagnostics Systems, Nutley, NJ) for cholesterol (18) and ceruloplasmin (19).

The lung, liver, heart, and kidney, as well as diet samples, were assayed for copper and iron content. Organs were lyophilized, digested with nitric acid and hydrogen peroxide, diluted in hydrochloric acid, and analyzed for mineral content by flame atomic absorption spectrophotometry (model 503; Perkin Elmer, Norwalk, CT) (20). The same procedure, except for lyophilization, was followed for diet samples. National Bureau of Standards reference samples were bovine liver (for organ analyses) and citrus leaves (for diet analyses). Measured mineral contents of reference samples were within the ranges specified.

Statistical Analysis. Two-way repeated measures analysis of variance (ANOVA) (21) was used to assess the effect of copper deficiency, the effect of concentration of inspired oxygen, and the interaction of the two variables on rate of breath ethane production. Other comparisons were by the Student's *t* test for unpaired means.

Results

Analysis of three samples of each diet indicated that the CuS diet had 5.1 ± 0.2 and the CuD diet had 0.2 ± 0.1 μg of copper/g diet. Iron content was 42.3 ± 0.9 and 43.3 ± 2.8 μg iron/g diet in CuS and CuD diets, respectively.

Characteristics of rats fed copper-sufficient (CuS) and copper-deficient (CuD) diets are shown in Table II. Direct evidence that copper status was reduced in CuD rats was the depressed copper content of the four organs examined. Also found were the characteristically

Table II. Characteristics of Rats Fed Copper-supplemented (CuS) and Copper-deficient (CuD) Diets

Variable[a]	CuS	CuD[b]
Body wt (g)	240 ± 33	177 ± 16
Heart wt (g)	1.1 ± 0.2	1.7 ± 0.5
Heart wt/body wt (mg/g)	4.4 ± 0.6	9.5 ± 2.6
Serum cholesterol (mg/dl)	84 ± 15	121 ± 21
Serum ceruloplasmin (mg/dl)	23.7 ± 9.7	5.6 ± 0.4
Liver copper (μg/g)	14.1 ± 3.7	1.5 ± 0.3
Liver iron (μg/g)	423 ± 167	649 ± 134
Lung copper (μg/g)	6.1 ± 0.7	3.2 ± 0.9
Lung iron (μg/g)	586 ± 157	304 ± 70
Heart copper (μg/g)	13.5 ± 2.5	3.2 ± 0.7
Heart iron (μg/g)	475 ± 60	271 ± 52
Kidney copper (μg/g)	17.5 ± 2.0	9.6 ± 0.9
Kidney iron (μg/g)	264 ± 33	159 ± 18

[a] Values of variables are mean ± SD; $n = 10$ for CuS, $n = 9$ for CuD data.
[b] All CuD variables listed are significantly different ($P < 0.005$) from the corresponding CuS variables by the Student's t test.

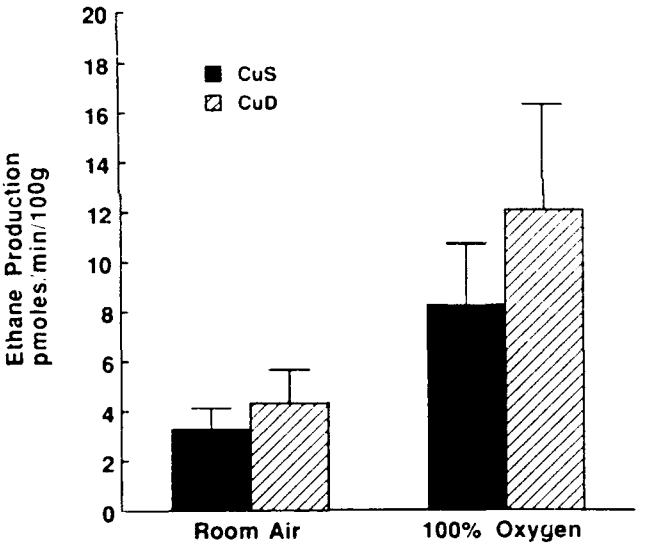

Figure 1. Ethane production (mean ± SD) measured in expired air of rats fed a copper-sufficient (CuS) or copper-deficient (CuD) diet when breathing room air or 100% oxygen. ANOVA indicates main effects of copper ($P < 0.01$) and oxygen content of inspired air ($P < 0.0001$), but no interaction effect of the two variables on ethane production.

higher liver iron content and lower iron content of other organs in CuD rats when compared with CuS rats. Rats fed the CuD diet had lower body weights, higher heart weights, higher serum cholesterol concentrations, and lower serum ceruloplasmin concentrations than CuS rats; all are signs previously associated with copper deficiency (22, 23).

The effects of copper deficiency and concentrations of inspired oxygen on breath ethane production are shown in Figure 1. Statistical analysis (repeated measures ANOVA) indicates that both copper deficiency ($P < 0.01$) and enhanced concentration of inspired oxygen ($P < 0.0001$) cause an increase in breath ethane production. The lack of an interaction effect indicates that these effects are independent of one another.

Discussion

In this study we have investigated the effect of dietary copper deficiency on breath ethane production. We used the protocol of Habib *et al.* (14), in which the ethane measurements on each rat were first made during air breathing and subsequently during breathing of 100% oxygen. We reconfirmed the findings of that study, which showed that increasing inspired oxygen content enhanced breath ethane production. We found however that the ethane production of control (CuS) rats in the present study was considerably lower than that of the previous study (14). We believe the difference may have been a consequence of the difference in diets (purified versus nonpurified diet) between the two studies, a suggestion supported by work which has shown that alteration of dietary fatty acid composition can influence exhaled amounts of the corresponding alkanes (24, 25).

Some questions arise as to the source of ethane in this study, given that our primary fat source is safflower oil, which contains primarily α-linoleic acid, an n-6 fatty acid. We have not assayed our diet for fatty acids; the fact that ethane is produced provides the strongest evidence that n-3 fatty acids are present in these rats. Possible sources of n-3 fatty acids as substrate for ethane production include the safflower oil itself, in which small amounts of n-3 fatty acids have been found (26), the casein in our basal diet, which contains trace amounts of fat (R. J. Rose, Teklad Test Diets, personal communication), or fatty acids incorporated from pre-experimental food sources consumed by the rats.

The major finding of this study was that dietary copper deficiency caused an increase in the rate of ethane exhaled. The most obvious, but not only, explanation of this finding is that a reduction in activity of copper metalloenzymes having antioxidative functions allowed enhanced activity of oxygen-derived free radicals and other reactive species. This would result in enhanced lipid peroxidation, including β-scission of n-3 fatty acids and resultant ethane production. Evidence exists which supports the occurrence of a reduced antioxidant enzyme activity in copper deficiency (1-3). As indicated in the introduction, positive, but rather limited and indirect, evidence exists indicating that enhanced lipid peroxidation occurs in copper-deficient animals *in vivo*. Our finding of enhanced breath ethane production provides additional support of a more direct nature for this hypothesis.

Our interpretation that enhanced breath ethane indicates enhanced lipid peroxidation must be conditioned by the observations that alkanes are catabolized

by the rat (27) and that alkane catabolism is subject to inhibition (28). If ethane catabolism were reduced by copper deficiency, breath ethane might be enhanced. This would require that the monooxygenase responsible for ethane catabolism be impaired by copper deficiency.

The possibility that reduced food intake of copper-deficient rats, as indicated by their lower body weight, caused the increased ethane production is refuted by the work of Habib *et al.* (29). They found that rats fed 60% of the amount of food consumed by groups of rats fed *ad libitum* actually had lower ethane production rates than the *ad libitum* group. This indicates that short-term food restriction (2 weeks) reduces lipid peroxidation as measured by ethane in the expirate and makes reduced food intake of the copper-deficient group an unlikely explanation for the increased ethane production observed.

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